Optimising biomarkers in cerebrospinal fluid

Willemse, E.A.J.

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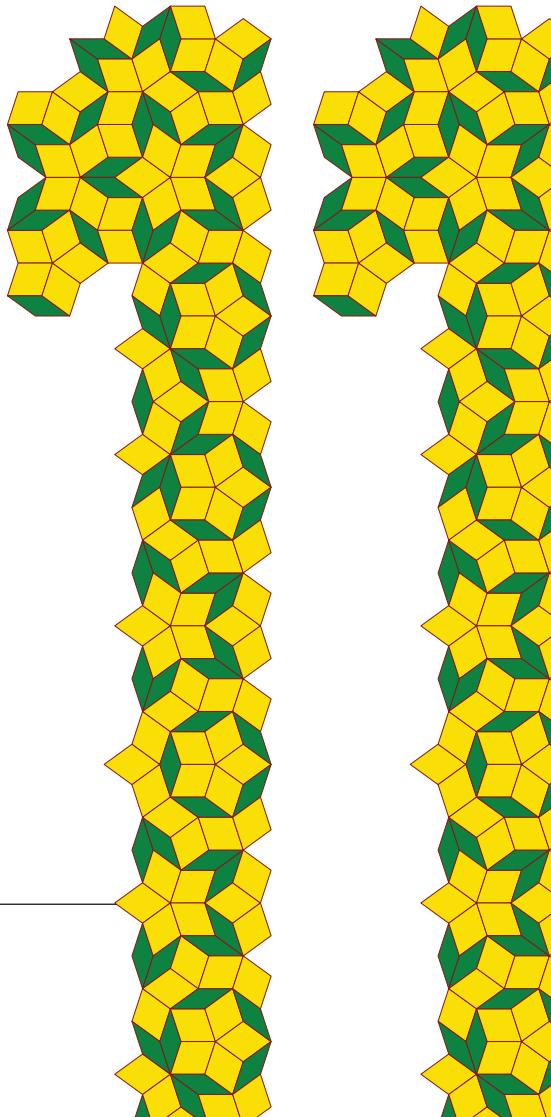
Summary and general discussion



General objective

The main aim of this thesis was to increase the reproducibility of CSF biomarker testing in order to improve the diagnosis for AD. To that end, the effects of pre-analytical and analytical confounders on CSF biomarkers were systematically analysed to optimise current biobanking collection and processing protocols and to increase the interpretability of CSF results, e.g. by providing stability results that could further facilitate clinical validation studies. Experimental evidence from this thesis shows that under various pre-analytical storage and freeze/thaw conditions and for the large majority of biomarkers tested, CSF is a rather stable matrix. We therefore endorse the current biobanking collection and processing protocol, as it was proven to preclude artificial concentration changes in any of the tested CSF biomarkers. In Box 1, the main recommendations for biomarker optimisation that resulted from this thesis are summarised. In assay comparison studies, performed for CSF progranulin, neurogranin, and $a\beta_{1\text{-}42}$, t-tau, and p-tau we observed that the absolute biomarker concentrations vary substantially when different assays were used to measure the same biomarker. This severely hampers the interpretation of results from one biomarker amongst research cohorts of patient samples tested with different assays. By direct assay comparison, as we performed for neurogranin, differences in the targeted epitopes of the same protein in different assays can be related to the CSF results reported by the different assays in the same clinical cohort. This direct translation between assays facilitates the next validation step in assay development, i.e. the clinical validation. Reproducibility of CSF biomarker testing can be increased through improvement of biomarker assays at various assay development stages. At an early stage of biomarker development, closer understanding of biomarker isoforms, conformational states of the biomarker in different matrices, or post-translational modifications of the biomarker is helpful for adequate design of sandwich immunoassays. Also, the availability of uniform reference material for the biomarker of interest, and the availability of a reference method, are needed in assay development to increase reproducibility of results. Later in the assay development stage, e.g. in the clinical implementation phase, analytical variation can be reduced by e.g. automation of biomarker assays.

Box 1. Main recommendations on (pre-)analytical factors in CSF to optimise biomarker studies resulting from this thesis.

Recommendations for pre-analytical processing factors in CSF:

- Maximum two hours delay between CSF withdrawal and centrifugation can be safely applied.
- Maximum two hours delay between CSF centrifugation and -80°C storage can be safely applied.
- Tube transfers of CSF should be avoided, especially for $a\beta_{1-42}$, or the $a\beta_{1-42}/a\beta_{1-40}$ ratio should be used to correct for $a\beta_{1-42}$ loss due to adsorption.

Recommendations for (pre-)analytical factors in CSF biomarker studies:

- Freeze/thaw cycles (tested up to 7) can be safely applied to the majority of CSF proteins tested, but should always be tested for novel biomarkers or novel methods.
- Long-term biobank storage at -80°C can be safely applied for the biomarkers $a\beta_{1-42}$, t-tau, p-tau, at least up to 12 years of storage.
- Evaporation does not occur in biobanking tubes stored at -20°C or -80°C, at least up to 4.5 years of storage.
- An unbiased statistical approach can correct for the upward assay drift of $a\beta_{1-42}$.
- Assay comparison studies help to translate results from the same biomarker measured using different assays, which facilitates further biomarker validation steps.
- The automated Elecsys $a\beta_{1-42}$, t-tau, and p-tau assays can be a good replacement for the manual Innotest $a\beta_{1-42}$, t-tau, and p-tau assays.

Summary of the findings

In **chapter 1** we delineated the context for this thesis by introducing international consensus guidelines for collection and storage procedures for CSF biobanks, thereby highlighting the issues for which more evidence is needed to support these guidelines. We further explained how biomarkers in CSF are essential for diagnosis and treatment development in AD, but that variability in CSF biomarker results hampers interpretation of these results. We therefore set out to systematically test pre-analytical and analytical confounders of CSF biomarker studies to increase the reproducibility of CSF biomarker studies in the subsequent chapters. In **chapter 2** we showed that evaporation does not occur during biobank storage at -20°C or -80°C for the (body) fluids CSF, plasma, serum, saliva, or water, for up to 4.5 years. This finding supports the use of historically collected patient cohorts stored in biobanks, as long-term storage is not compromised by evaporation of the samples. In **chapter 3** we concluded that the CSF

biomarker concentrations for $a\beta_{1-42}$, t-tau, and p-tau did not change upon -80°C biobank storage for up to 12 years. Our study design assumed that the mean biomarker concentrations in an homogeneous AD patient cohort would remain constant on group level over time, thereby allowing measurement of the biomarkers in patient samples with different storage times (2 – 14 years) while excluding assay batch interference. Although biomarker concentrations, measured using one assay batch, remained stable regardless of the biobank storage time, the originally reported $a\beta_{1-42}$ concentrations of the historical samples revealed that the $a\beta_{1-42}$ assay shows an upward drift over the years. This finding confirms that historical samples remain appropriate for biomarker studies, however, historically measured $a\beta_{1-42}$ results should be corrected for their year of measurement or should be remeasured. In **chapter 4**, we developed an unbiased, data-driven statistical model to correct the CSF $a\beta_{1-42}$ biomarker results from our Amsterdam Dementia Cohort (4397 patient samples) for the upward drift in CSF $a\beta_{1-42}$ concentrations over the last two decades. We verified the statistically corrected concentrations with the remeasured concentrations in a sub-cohort. This model for drift corrections is a useful tool to standardize CSF $a\beta_{1-42}$ biomarker data over time and amongst different centres or platforms.

In the next chapters, we zoomed in on the effects of pre-analytical processing steps on the CSF biomarker concentrations. In **chapter 5**, we found that CSF $a\beta_{1-42}$ concentrations were reduced during tube transfers of CSF, with a 5% decrease on average per transfer, of which half of the $a\beta_{1-42}$ concentration decreased due to pipetting only. We recommended to restrict the number of transfers during CSF work-up and to use the ratio $a\beta_{1-42}$/ $a\beta_{1-40}$, as this ratio was not affected by the variation caused by adsorption to lab plastics. In **chapter 6** we tested the stability of 11 novel CSF biomarker candidates under standardised pre-analytical conditions that included storage at room temperature or 4°C up to one week, or at -20°C up to 1 month, or up to 7 freeze/thaw cycles. One metabolite, MHPG, which is the principal metabolite of the neurotransmitter norepinephrine, showed a strong and linear decrease in concentration upon storage conditions and after f/t cycles. However, the other biomarkers did not change under these conditions, suggesting that these biomarkers can be trustfully tested under the pre-analytical conditions present across different cohorts. In **chapter 7** we extended this analysis to a larger range of proteins. Two array-based biomarker discovery platforms were used, SOMAscan and Olink, detecting both about a thousand proteins per sample. We defined stability criteria for the individual proteins based on the confidence interval of the difference between two extreme storage of freeze/thaw conditions. We found that >70 % of the proteins remained stable under the most extreme conditions of one week storage at room temperature or after 7 freeze/thaw cycles compared to the reference sample. This indicates that the large majority of CSF proteins remains stable when exposed to time delays between processing steps up to one week, or multiple freeze/thaw cycles. In **chapter 8** we demonstrated that concentrations

of the potential biomarker progranulin remained stable in serum and CSF when samples were exposed to standardised pre-analytical conditions. Additionally, we showed that progranulin levels measured with two different commercial immunoassays correlated well, indicating that this biomarker can be further validated in clinical samples. In **chapter 9** we compared three different immunoassays for the potential CSF biomarker neurogranin. We tested their epitope affinities and compared the absolute neurogranin levels in the same CSF samples of AD, FTD, VaD, DLB patients and control subjects. Although the absolute concentration ranges differed amongst the assays, the discriminative power of the assays amongst the clinical groups was comparable. The AD group presented with the highest neurogranin levels, but we found limited power for neurogranin as a differential diagnosis biomarker for dementia. In **chapter 10** we found high concordance in CSF biomarker results measured with the automated Elecsys assays and the routine manual Innotest assays for $a\beta_{1-42}$, t-tau and p-tau. CSF samples had been prospectively collected in local memory clinics in the Netherlands and thus closely reflect a real-life diagnostic setting. Results support the transition from the manual to the automated assays for the AD CSF biomarkers for clinical routine in research setting.

In this thesis we deciphered crucial factors of pre-analytical and analytical variation to increase awareness on how these factors influence the reproducibility of CSF testing, as well as how to manage these variation factors in order to increase reproducibility (Box 1). For CSF processing, we found that time delay between processing steps was not influencing biomarker concentrations, but that tube transfers of CSF should in principal be avoided. As for the pre-analytical factors in CSF biomarker studies, we found that long-term storage (up to 12 years) did not affect the AD biomarkers, nor did additional freeze/thaw cycles (up to 7), although storage and freeze/thaw stability testing should be applied to novel biomarkers and novel methods. Furthermore, we showed that the upward assay drift of $a\beta_{1-42}$ over the last years can be statistically corrected for. For routine CSF AD biomarker measurements, we found that the recently developed fully automated Elecsys assays showed high concordance with the manual Innotest assays, thereby supporting the transition from manual to automated tests. Direct assay comparison studies for CSF biomarkers presented in this thesis helped the translation of results amongst the different assays, which facilitates further development of the biomarkers towards clinical implementation. Overall, increased reproducibility of CSF biomarker testing will support the clinical implementation of AD diagnostic biomarkers and facilitates the development of novel biomarkers for AD and other neurodegenerative diseases.

Recommendations for CSF stability testing

Next to the recommendations for increased reproducibility of CSF biomarker studies (Box 1), several tools have been developed in the course of this thesis. These applications were partly

based on the results of this thesis and were designed to provide hands-on tools that facilitate the harmonisation of biomarker studies. These tools are broadly available to the CSF biomarker and assay development field and will contribute to successful development of clinical CSF biomarkers.

First, the **self-assessment tool** of the International Society for Biological and Environmental Repositories (ISBER) for evaluating the biobanking quality for blood, tissue, and cells, has been expanded with questions on the pre-analytical handling of CSF. These extra questions were based on the results reported in this thesis on the pre-analytical processing effects on CSF, and related to processing and storage times and temperatures during CSF work-up. The self-assessment tool tests the compliance of the biobank to best practices, and thereby stimulates the harmonisation of biobank collection, processing and storage procedures that are the basis to perform reproducible biomarker studies [1].

Second, the **standard operating procedure (SOP) for stability testing**, which is part of broader guidelines for immunoassay validation [2], defines systematic exposure of CSF samples to pre-analytical conditions as performed in chapters 6 and 7 of this thesis. We additionally developed a tool, tailored to this stability SOP, that facilitates a standardised way of reporting the stability results. This **biospecimen stability testing calculator, 'stabcalc'**, is openly available online (http://www.isber.org/?page=STABCALC) and was approved by the ISBER. The stability SOP and STABCALC together facilitate a uniform way of testing and presenting stability results and are therefore valuable tools to increase the reproducibility of biomarker studies.

Third, the Integrated Biobank of Luxembourg (IBBL) runs an annual biobank **proficiency testing scheme** to compare performances of biobank sample work-up, such as aliquoting, amongst laboratories. Proficient biobanks subsequently get certified and accredited [3], which increases the quality of biobank samples and increases reproducibility of biomarker studies.

Fourth, an **external quality control testing programme** has been established at the University of Gothenburg in Sweden, supported by the Alzheimer's Association. This quality control programme focusses on the AD CSF biomarkers specifically and compares different assays for the same biomarkers amongst different laboratories [4]. This proficiency testing programme reports on the reproducibility of the AD CSF biomarker assays and helps to trace the source of the assay- or laboratory-related variability in biomarker results.

These tools are valuable efforts to reduce pre-analytical and analytical variation and will together result in increased reproducibility for biomarker studies in CSF.

Towards clinical implementation of the diagnostic AD CSF biomarkers

The recent release of automated assays to measure CSF $a\beta_{1-42}$, t-tau, and p-tau significantly reduces the variability in biomarker results, resulting in a total reproducibility of 2.0-5.1% [5]. One

of the main factors that caused the large variability of the manual immunoassay, in particular for $a\beta_{1-42}$, was the different assay composition between batches, as reported in **chapter 3 and 4**. Another influential variation factor for $a\beta_{1-42}$ is its tendency to adhere to surfaces, such as lab plastics, which was studied in **chapter 5**. The automated pipetting and transfer steps of the robotic assays will greatly contribute to a reduction of the variation of biomarker results compared to manual pipetting. Another benefit of the automated assays with regard to clinical implementation is that they allow high throughput measurements and direct analysis of fresh samples, without the need of transfer to a new tube and intermediate storage. In **chapter 10** we showed that the automated assays for AD CSF biomarkers gave similar results in a real-life diagnostic setting compared to the manual immunoassays. Automation, and the calibration of these assays against certified reference material, yielded a closely comparable cut-point for $a\beta_{1-42}$ amongst three different cohorts to discriminate AD patients from a non-AD profile [6,7]. These developments suggest that the field is approaching the establishment of a universal cut-point for AD CSF biomarkers, which will be followed by global clinical implementation of these assays for AD diagnosis.

An alternative to solve the variability of CSF biomarker results is a computational approach. CSF $a\beta_{1-42}$ concentrations of a random population typically show a bimodal distribution that reflects pathological and non-pathological biomarker levels. Systematic variations in biomarker levels, e.g. between laboratory A and B, can be corrected for by shifting the distributions to the optimal overlap of both populations. In research settings this data-driven approach helped to correct for $a\beta_{1-42}$ assay drift as shown in **chapter 4**. This data-driven statistical correction is a robust method to align cohorts, but can also be applied to align different methods. This so-called Gaussian mixture modelling can be used for a large range of biomarkers as long as the sample size is sufficient to estimate the distribution pattern – preferably n > 800 for an accurate estimation. This computational approach to compare biomarker studies can thus be broadly applied to increase reproducibility.

Potential novel biomarkers in AD biomarker research

Although CSF $a\beta_{1-42}$, t-tau, and p-tau are approaching clinical implementation as biomarkers for AD [8,9], the quest for novel biomarkers proceeds, as the needs for differential diagnosis biomarkers, biomarkers that reflects disease progression, and biomarkers for axonal damage remain compelling.

We and others found elevated levels of CSF neurogranin in AD versus other forms of dementia (**chapter 9**; [10–12]), however, we concluded that CSF neurogranin is not suitable as a potential differential diagnosis biomarker for AD dementia due to its high correlation with CSF t-tau and p-tau. Nonetheless, neurogranin does seem promising as a monitoring biomarker for AD since it

reflects the cognitive decline and thus could be used as surrogate outcome in clinical trials [13,14]. Also, neurogranin levels seem to increase at very early stage, which fits the hypothesis that AD pathology starts with synaptic dysfunction [15,16]. To develop a biomarker test for neurogranin as a monitoring biomarker, the relation of neurogranin to cognitive decline should be further studied in an appropriate cohort to develop relevant reference values. In **chapter 9** we compared three assays for neurogranin and defined different epitopes of neurogranin that were detected by these assays. We found that although the three assays targeted different epitopes of the neurogranin protein, the neurogranin results measured in a patient cohort of dementia differential diagnoses were highly comparable, showing increased neurogranin concentrations in AD patients. This background can be taken advantage of to further develop neurogranin as a clinical biomarker.

Several novel CSF biomarkers have been validated that could successfully discriminate AD patients from controls, however, none of those significantly increased diagnostic power to the current AD biomarker panel of $a\beta_{1\text{-}42}$, t-tau, and p-tau. For differential diagnoses of AD, a few novel biomarkers have been discovered that are used for diagnosis in research settings. YKL-40 is a glycoprotein present in astrocytes of which CSF levels are increased in AD compared to controls [17–20]. However, CSF YKL-40 levels are also increased in other neurodegenerative diseases, such as Creutzfeldt-Jakob disease (CJD), and are specifically reflecting neuroinflammation [21,22]. Neurofilament light (NfL) is an axonal marker reflecting neurodegeneration in CSF that shows elevated levels in AD patients compared to controls, but shows also elevated levels in other neurodegenerative diseases [23–26]. Moreover, NfL in serum and plasma similarly showed increased levels in AD patients compared to controls with a diagnostic power comparable to the CSF AD biomarker panel [27,28]. However, plasma NfL levels were also increased in other neurodegenerative diseases such as FTD and progressive supranuclear palsy [29,30]. As a marker for axonal injury, NfL in blood shows high potential as biomarker for many diseases, also beyond the field of neurodegenerative diseases, such as acute cardiovascular events and amyotrophic lateral sclerosis [31,32]. In conclusion, more CSF biomarkers are discovered that specifically reflect a brain pathology, such as aβ-pathology, axonal damage, or neuroinflammation, and this will help us to understand and find new diagnostic biomarkers for the different types of neurodegenerative diseases.

As a diagnostic screening tool, biomarkers in blood have large advantages over biomarkers in CSF. The discovery of AD biomarkers in blood has been difficult because CNS proteins have much lower concentration in blood than in CSF and thus could not be detected by the methods available at that time. In the last years, novel instruments have been developed that perform ultrasensitive measurements, such as the immuno-magnetic reduction (IMR) and Single-molecule array (Simoa) methods, that succeeded to detect AD biomarkers in blood [33]. Increased levels of plasma tau in AD compared to controls have been found, although with large

overlap between groups [34–36]. Longitudinal measurements of plasma tau showed an association with biomarkers of disease progression, including increased brain atrophy, worse cognition, and brain hypometabolism [36,37]. Also NfL, in either plasma or serum, has recently shown as successful biomarker for axonal damage in many neurological or cerebrovascular diseases, as discussed in the previous paragraph. Thus, due to the development of novel ultrasensitive measurement techniques, diagnostic tools for AD biomarkers in blood are emerging.

As blood contains more cells, enzymes, etc. than CSF, it is generally expected that blood biomarkers are more susceptible to changes under pre-analytical conditions than the CSF biomarkers. Blood protein levels are particularly more prone to fluctuate upon e.g. diet or other patient-related factors, so these patient related pre-analytical factors should be considered carefully during biomarker validation. Blood-based biomarkers can be screened for stability using the same SOP for stability testing as was recommended for CSF, extended with patient-related pre-analytical variation factors, including diet and diurnal rhythm.

Conclusion

Studies aimed to increase the reproducibility of laboratory tests for AD CSF biomarkers, as presented in this thesis, led to:

1) More insight in the effects of pre-analytical variation on biomarker concentrations and disease classifications:

- Long-term storage of CSF samples does not cause evaporation of the samples, nor does it affect the concentrations of AD biomarkers
- Most CSF biomarkers tested in this thesis are stable under common pre-analytical variation factors such as additional freeze/thaw cycles and time delays during sample processing.
- The upward assay drift observed for Innotest $a\beta_{1\text{-}42}$ over the last decade can be statistically corrected for in order to harmonise $a\beta_{1\text{-}42}$ results obtained over time.

2) Experimental evidence from this thesis supports the recommendations in the current guidelines for CSF biobank collection and processing with regard to processing times and temperatures. With regard to transferring CSF from tube to tube, results from this thesis indicate that this pre-analytical handling should be avoided as it significantly reduced the concentration of $a\beta_{1\text{-}42}$.

3) Through assay comparison studies we achieved a better interpretation of results from different assays for the same novel biomarker candidates, e.g. progranulin and neurogranin. The assay comparison for neurogranin proved that the targeted epitopes of three assays were different but CSF concentrations amongst clinical groups were comparable. Comparison of novel AD biomarker assays showed high concordance between fully automated Elecsys and manual Innotest assays for $a\beta_{1\text{-}42}$, t-tau, and p-tau.

All three outcomes are essential to realise the transition from CSF biomarker tests used for research purposes in specialised centres to CSF biomarker tests that are suitable for general use in clinical practice. The reproducibility tests described in this thesis, e.g. stability under (long-term) storage and freeze/thaw conditions, and assay comparison studies, pave the way to define global cut-points for the established CSF biomarkers $a\beta_{1\text{-}42}$, t-tau, and p-tau. For novel CSF biomarkers for AD, these reproducibility tests enable robust interpretation of results, resulting in adequate evaluation of the potential of these markers in AD or related types of dementia.

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Appendices